

FOOD AND BEVERAGE MYCOLOGY

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Metabolites of Fungi Used in Food Processing

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Fungal metabolites have traditionally been a part of food preparation. Fermented foods, based on the ancient koji process, are staples in Oriental diets. The Western world is familiar with the role of fungi in the ripening of cheeses such as Roquefort and Camembert, and fermented beverages have been consumed for centuries throughout the world. No attempt will be made in this chapter to review the historic role of fungi in food processing, since this topic is covered rather extensively in Chap. 6, 9, and 10. Instead, an attempt will be made to show the role of fungal metabolites in modern food processing. The modern era was initiated in the 1920s after Thom and Currie discovered in 1916 that *Aspergillus niger* formed large amounts of citric acid from sucrose. About the same time, and as a result of the work of Takamine, the fungal enzyme industry was established. From this start the fermentation industry has expanded so that today several organic acids and a variety of enzymes produced by fungi are used in the manufacture of foods and beverages. In addition, fungal fermentations are conducted to make amino acids, vitamins, flavoring agents, mannitol, and fats and oils, all of which are of value in food processing.

ORGANIC ACIDS

Organic acids function primarily as acidulants in modern food processing. In addition to rendering foods more palatable and stimulating to the consumer, Gardner (1968) has listed the following uses for acidulants.

(1) Flavoring agents, where they may intensify certain tastes, blend unrelated taste characteristics, and mask undesirable aftertastes.

(2) Buffers, in controlling the pH of food during various stages of processing, as well as of the finished product.

(3) Preservatives, in preventing growth of microorganisms and the germination of spores which lead to the spoilage of food or cause food poisoning or disease.

(4) Synergists to antioxidants, in preventing rancidity and browning.

(5) Viscosity modifiers, in changing the rheological properties of dough and, consequently, the shape and texture of baked goods.

(6) Melting modifiers, for such food products as cheese spreads and mixtures used in manufacturing hard candy.

(7) Meat curing agents, together with other curing components in enhancing color, flavor, and preservative action.

Only the fungal-produced organic acids used in food processing will be covered in this chapter, and discussion will be confined largely to use and production. A list of these organic acids is shown in Table 13.1.

Citric Acid

Uses.—Citric acid is widely used in the preparation of soft drinks, cheeses and other dairy products, desserts, jams, jellies, candies, canned seafoods, wines, and frozen fruits. Citric acid is also used in gelatin food products and with artificial flavors of dry compounded materials such as soft drink tablets and powders. It functions as an antioxidant for inhibiting rancidity in fats and oils, and as an acidulant, buffer, emulsifier, and stabilizer in various food products.

Production by Molds.—Citric acid was first made commercially in England in 1860 from calcium citrate obtained from cull lemons in Italy and Sicily (Lockwood and Schweiger 1967). In 1922, Italy produced approximately 90% of the world's supply of citrates (Prescott and Dunn 1959). The introduction of mold-produced citric acid (Currie 1917) in the United States in 1923 broke this monopoly, and now most of the world's supply of citric acid is produced by carbohydrate fermentation.

The microorganisms most commonly used in the production of citric acid are selected strains of *Aspergillus niger* (Fig. 13.1). However, other molds such as *A. wentii*, *A. clavatus*, *Penicillium luteum*, *P.*

TABLE 13.1
FOOD ACIDS PRODUCED BY FUNGI

Acid	Formula	Produced By	Food and Beverage Use
Citric	$ \begin{array}{c} \text{H}_2\text{C}-\text{COOH} \\ \\ \text{HO}-\text{C}-\text{COOH} \\ \\ \text{HO}-\text{C}-\text{COOH} \\ \\ \text{H}_2\text{C}-\text{COOH} \end{array} $	<i>Aspergillus niger</i> , <i>Candida lipolytica</i>	Soft drinks, dairy products, desserts, jams, jellies, candies, canned seafoods, wines, frozen fruits, fats, and oils
Itaconic	$ \begin{array}{c} \text{H}_2\text{C}=\text{C}-\text{COOH} \\ \\ \text{H}_2\text{C}-\text{COOH} \end{array} $	<i>Aspergillus itaconicus</i> , <i>A. terreus</i>	Shortenings, resin coatings contacting food
Gluconic	$ \begin{array}{c} \text{COOH} \\ \\ \text{H}-\text{C}-\text{OH} \\ \\ \text{HO}-\text{C}-\text{H} \\ \\ \text{H}-\text{C}-\text{OH} \\ \\ \text{H}-\text{C}-\text{OH} \\ \\ \text{H}_2\text{C}-\text{OH} \end{array} $	<i>A. niger</i> , <i>Penicillium chrysogenum</i>	Bottle washing formulations, baking powder, bread mixes, emulsified meat products, desserts
Fumaric	$ \begin{array}{c} \text{H}-\text{C}-\text{COOH} \\ \\ \text{H}-\text{C}-\text{COOH} \end{array} $	Species of <i>Rhizopus</i> and <i>Mucor</i>	Fruit drinks, desserts, wines, doughs, coatings, fats, dairy and meat products

Malic	$ \begin{array}{c} \text{H}_2\text{C-COOH} \\ \\ \text{H-C-COOH} \\ \\ \text{OH} \end{array} $	Species of <i>Aspergillus</i> , <i>Penicillium brevi-compactum</i> , yeasts	Beverages, jams, jellies, candy, syrups, sour dough, oils
Tartaric	$ \begin{array}{c} \text{COOH} \\ \\ \text{H-C-OH} \\ \\ \text{HO-C-H} \\ \\ \text{COOH} \end{array} $	<i>Penicillium notatum</i> , <i>A. niger</i> , <i>A. griseus</i>	Carbonated beverages, desserts, jellies
Succinic	$ \begin{array}{c} \text{H}_2\text{C-COOH} \\ \\ \text{H}_2\text{C-COOH} \end{array} $	Species of <i>Rhizopus</i> , <i>Mucor</i> , <i>Fusarium</i>	Flavorings
Oxalic	$ \begin{array}{c} \text{COOH} \\ \\ \text{COOH} \end{array} $	<i>A. niger</i>	Hydrolysis of starch to glucose
Lactic	$ \begin{array}{c} \text{CH}_3 \\ \\ \text{H-C-OH} \\ \\ \text{COOH} \end{array} $	Species of <i>Rhizopus</i> and <i>Mucor</i>	Fruit juice, shortenings, mayonnaise, mincemeat, desserts, bakery, dairy, and meat products

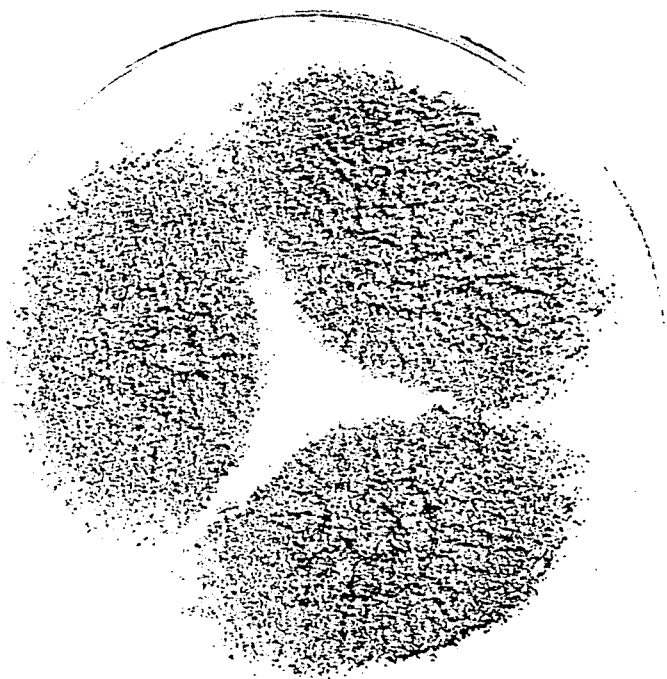


FIG. 13.1. *ASPERGILLUS NIGER* NRRL-334 CULTURED ON CZAPEK AGAR FOR 10 DAYS AT ROOM TEMPERATURE

citrinum, *Paecilomyces divaricatum*, and *Mucor* spp. have been used in the laboratory

A surface culture process or shallow pan method and a submerged culture method are used in the commercial production of citric acid. Both fermentations rely on interruption of the Krebs citric acid cycle in a terminal stage of carbohydrate metabolism. In the surface culture method, a solution of suitable carbohydrate is inoculated with spores of *A. niger* and poured into shallow aluminum or stainless steel pans. The carbohydrate may be refined or crude sucrose, fructose, glucose, high-test cane syrup, or beet molasses. In general, sugar concentrations of 20 to 25% are required to produce high yields of citric acid. The high sugar concentrations are believed to inhibit the formation of acids other than citric, and the addition of ferricyanide (Clark and Lentz 1963) followed by filtration is reported to reduce the dissolved iron which interferes with citric acid accumulation (Schweiger 1961). Molliard (1922) showed that phosphate deficiency enhanced citric acid production, and undoubtedly the presence or absence of trace

amounts of other elements in the medium may have a marked effect on the fermentation. Doelger and Prescott (1934) indicated that a pH of 1.6 to 2.2 is optimum for citric acid production. By balancing ammonium salts of mineral acids and alkali metal nitrates, the fermentation can be controlled to give only citric acid. Maximum acid production occurs when the rate of growth of mycelium is insignificant and there is no sporulation. During the first 5 to 7 days of fermentation, humidified air at 28° to 34°C is blown across the surface of the culture. After 8 to 10 days the solution is drained off and the mat is washed and pressed to remove any acid contained in it. Calcium citrate is then precipitated from a hot neutral solution. Next a sulfuric acid treatment is used to liberate citric acid. Usually about 70% of the weight of the sugar used in the medium can be recovered as citric acid (Schweiger 1961).

The submerged culture method of citric acid production has developed since 1930. The studies of Shu and Johnson (1947, 1948A, B) showed that the fermentation was quite sensitive to manganese and iron. Moyer (1953) demonstrated that methanol and ethanol stimulate production of citric acid by *A. niger*. However, it was not until (1) methods were developed for removal of metallic ions with ion exchange resins (Woodward et al. 1949), (2) copper was used as an antagonist for ions (Schweiger 1961), and (3) suitable strains of *A. niger* were selected that the present commercial process developed. Decationized solutions of high-test syrup, glucose, or sucrose are suitable for use as carbohydrate sources (Lockwood and Schweiger 1967). Except for ammonia, which is used to adjust the initial pH in the range of 2 to 4, nutrients are added similarly to the surface culture process. However, restriction of phosphate is unnecessary and air is bubbled through the fermentation solution at a rate of 0.5 to 1.5 (vol/vol/min) (Lockwood and Schweiger 1967). The fermentation is usually conducted over a 5- to 14-day period at 27° to 33°C. The acid is then harvested by a method similar to the one used in the surface process.

Production by Yeasts.—The commercial process for production of citric acid with *A. niger* has several disadvantages. For example, with time the citric acid-producing capability of the *A. niger* culture tends to degenerate, and more than 7 days are required for maximum acid production. Thus, it is obvious that the development of a rapid fermentation process is of considerable commercial importance. A typical process flow sheet is given in Fig. 13.2.

Over the last 10 years, several yeast strains of the genera *Candida* (perfect stage—*Saccharomycopsis*), *Endomycopsis*, *Torulopsis*, *Hansenula*, and *Pichia* have been studied that have the ability to accumulate substantial amounts of citric acid during the aerobic

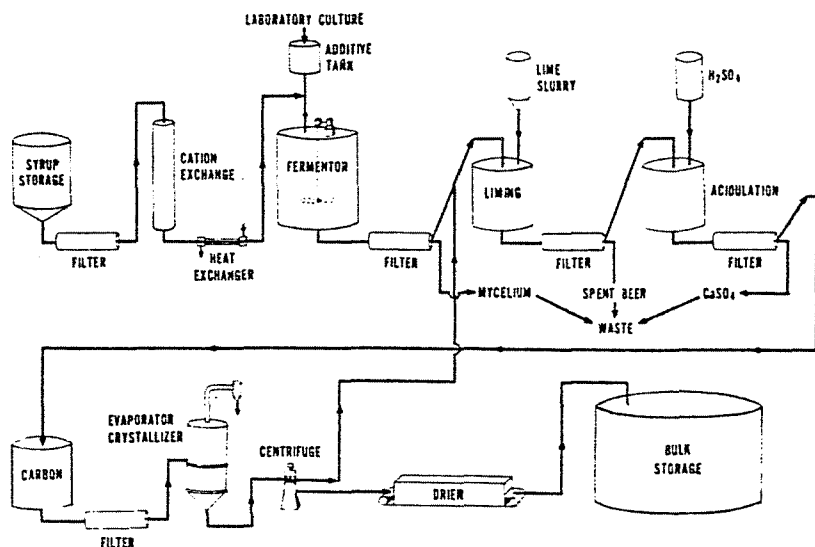


FIG. 13.2. A TYPICAL PROCESS FLOW SHEET FOR CITRIC ACID

fermentation of aqueous carbohydrate, hydrocarbon, and/or acetic acid-containing media (Roberts 1973; Hustede and Siebert 1974; Takayama and Tomiyama 1974; Furukawa and Kaneyuki 1975). Roberts (1973) found that yeasts will accumulate larger amounts of citric acid when halogen-containing agents (α -chloro- and α -fluoro-substituted lower alkanolic mono- and dicarboxylic acids) are included in the fermentation medium. A major problem with yeasts is that many strains gradually consume the citric acid produced during fermentation and for this reason the yield is reduced. Fukuda *et al.* (1974) emphasized the need for selecting strains of yeasts that are capable of utilizing hydrocarbons and incapable of using citric acid. Hustede and Siebert (1974) produced 128 g of citric acid/liter of hydrocarbon-containing medium in 4 days with a mutant strain of *Candida oleophila*. Furukawa and Kaneyuki (1975) reported yields of 150 g/liter in 7 days with *Saccharomycopsis lipolytica* on a hydrocarbon medium.

Ikeno *et al.* (1975) have recently studied citric acid production by mutant strains of *Candida lipolytica* on natural oils, fatty acids, glycerol, ethanol, and *n*-paraffin. They reported 102 g/liter of citric acid from palm oil in 94-hr fermentations and yields of citric acid against oil of 146%. On *n*-paraffin, yields of 170 g/liter were obtained. Although the potential for economical production of citric acid by yeasts exists, commercial production is still in the developmental stage.

Itaconic Acid

Uses.—Itaconic acid is principally used in resin coatings for paper. It offers superior properties in taking printing inks and in bonding. Food-related uses include itaconic acid-modified triglycerides as shortening compounds and the use of itaconic copolymers in coatings which come into contact with foods.

Production.—Itaconic acid was first reported by Kinoshita (1929) to be a fungal metabolite of *Aspergillus itaconicus*. Later Calam et al. (1939) found that certain strains of *Aspergillus terreus* produced significant amounts from glucose. Further studies (Lockwood and Reeves 1945; Moyer and Coghill 1945; Lockwood et al. 1945) led to the isolation of a superior strain of *A. terreus* (NRRL 1960) for the production of itaconic acid in surface and submerged culture. Subsequent developmental work by Lockwood and Nelson (1946), Pfeifer et al. (1953), and Batti and Schweiger (1963) led to the current process for itaconic acid production which is similar to the one described by Nubel and Ratajak (1962). In this process, spores of *A. terreus* are germinated 18 hr in a beet molasses medium. A 15% cane molasses medium containing zinc sulfate, magnesium sulfate, and cupric sulfate is then inoculated with a 20% (vol/vol) spore suspension. Air is bubbled through the solution, vigorous agitation is applied, and the temperature is maintained at 39° to 42°C. The pH drops from 5.1 to about 3.1 during the first 24 hr. Lime or ammonia is then used to readjust the pH to 3.8 and the fermentation continues for 2 more days. At the end of the fermentation, the mycelium is filtered off and washed, and the solution is concentrated by heating. The hot solution is decolorized with carbon, and on cooling the itaconic acid crystallizes. Alternatively, a calcium precipitation similar to one described for citric acid may be used. Yields of itaconic acid are about 85 g/liter. As with the citric acid fermentation, the itaconic acid fermentation is most efficient below pH 2 and when the mycelium growth rate is insignificant. The fermentation is also sensitive to metallic ions.

Gluconic Acid

Uses.—Many important applications have been found for gluconic acid and its sodium, calcium, and lactone derivatives in the food industry. The largest markets for gluconates are in alkaline bottle-washing formulations used in cleansing milk, beer, and soft drink bottles. Favorable properties of gluconates include ease of handling, low toxicity, low corrosion of metals, good cleaning efficiencies, compatibility with aqueous systems, and prevention of calcium, magnesium, and iron deposits (Ward 1967). The latent acidity of δ -gluconolactone has led to its use as a component of baking powder, in which it is not reactive with sodium bicarbonate until water is added. This property along with edibility makes δ -gluconolactone desirable

for use in chemically leavened bakery goods and bread mixes (Feldberg 1959). Also, because of its latent acidity this compound has produced excellent results in the controlled acidification and retention of red color in emulsified meat products (Sair 1961). This same latent acid property has been applied to cheesemaking by using the lactone as an acidogen before curd formation (Hammond and Deane 1961). Bland-flavored sherbets (Feldberg 1959) and cold milk puddings (Hunter and Rock 1960) have been prepared using δ -gluconolactone as a latent acidulant. Also the lactone has been used to reduce fat absorption in rolled waffle cones and doughnuts (Feldberg 1959).

Production.—Early attempts to produce gluconic acid by the surface cultivation of *Penicillium* and *Aspergillus* spp. were made by Herrick and May (1928) and May et al. (1929). May et al. (1934) and Gastrock and Porges (1938) later applied submerged cultivation techniques and calcium carbonate neutralization to gluconic acid production. Subsequent studies by Moyer et al. (1937), Wells et al. (1937), and Blom et al. (1952) on glucose concentration, agitation, aeration, and air pressure laid the groundwork for the production of gluconates by submerged growth of *Aspergillus niger* as it is practiced today. A glucose concentration of 30%, temperature maintained at 33° to 34°C, fermentors of several thousand liters capacity, aeration rates of 1.0 to 1.5 vol of air per min per vol of fermentation broth, air pressure on the fermentor of 30 psig (about 2900 g/cm²), and a high degree of agitation are preferred for gluconic acid (sodium salt) production (Ward 1967). Theoretical yields of 95% are obtained. Fermentors can be inoculated with either spore suspensions or vegetative mycelium, or with mycelium separated from a previous fermentation. Calcium or sodium salts of gluconic acid are recovered by incorporating calcium carbonate or sodium hydroxide in the culture medium. Gluconic acid may be recovered from calcium gluconate broths or solutions by sulfuric acid treatment or from aqueous sodium gluconate solutions by ion exchange. The lactones are recovered by temperature-critical crystallization of oversaturated gluconic acid solutions. Below 30°C gluconic acid is recovered. From 30° to 70°C, the δ -gluconolactone is predominant, and above 70°C the γ -lactone is obtained (Pasternack and Giles 1934).

Fumaric Acid

Use.—Fumaric acid is used in fruit juice drinks, gelatin desserts, pie fillings, refrigerated biscuit doughs, maraschino cherries, and wines (Gardner 1968). Fumaric acid is useful in preparing edible coatings for candy, water-in-oil emulsifying agents, reconstituted fats, and dough conditioners (Bertram 1951). It also shows good antioxidant properties in lard, butter, cheese, powdered milk, sausage, bacon, nuts, and potato chips (Gardner 1968) and may be used for improving

the whipping properties of gelatin and egg white (Conrad and Stiles 1954; Abbott et al. 1957). Fumaric acid is economical from the standpoint of cost and the quantities required for imparting acid tastes; however, its application is limited for some purposes because it goes into solution slowly and has a relatively low solubility in water (Gardner 1966).

Production.—Fumaric acid is produced principally by the fermentation of glucose or molasses with species of the genus *Rhizopus*. Foster (1949, 1954) has reviewed production of fumaric acid by these fungi. The level of various components of the fermentation medium such as carbohydrate, potassium, iron, magnesium, zinc, and copper can greatly influence the yield. Only under limited conditions do selected strains of *Rhizopus* produce primarily fumaric acid. Usually ethanol is produced with substantial proportions of other acids. Rhodes et al. (1959) reported fumaric acid yields of 60 to 70% in 3 to 8 days in shaken flasks containing 10 to 16% concentrations of glucose, sucrose, or the partially inverted sucrose of high-test molasses. Of the total acids produced, 75 to 80% was fumaric acid. Up to 25% greater yields of fumaric acid were obtained by the addition of methanol to fermentations conducted with molasses.

Even though fumaric acid can be produced in rather good yields by fermentation, the economics today are such that fumaric acid is produced commercially as a by-product in the manufacture of phthalic and malic anhydrides or by isomerization of malic acid with heat and a catalyst (Dmuchovsky and Franz 1967).

Malic Acid

Use.—Malic acid is a general purpose acidulant. It has unusual taste-blending characteristics and in some instances appears to have flavor-fixing qualities, as well as serving to overcome undesirable aftertastes (Gardner 1966). Apparently malic acid has a stronger acidic taste than does citric but not as strong as fumaric.

The United States Food and Drug Administration (1965) has included malic acid as a miscellaneous and/or general-purpose food additive in its list of GRAS substances. Malic acid has been used in the manufacture of apple jams, jellies, candy, and beverage products. However, the use of malic acid in food has been limited to date (Pederson 1971). Applications have been restricted primarily to specialty items such as a peach drink, confectionery products, apple-flavored hard candy, ice-cream syrups, and tobacco products (Irwin et al. 1967). Malic acid has been employed in extracting pectin, in the production of sour dough, and in the synthesis of emulsifying agents which inhibit the development of rancidity in oils (Gardner 1968). Pray and Powers (1966) used malic acid as an acidifying agent in canning tomatoes.

Production.—Malic acid has been found in cultures of a variety of fungi, including aspergilli, yeasts, and *Penicillium brevi-compactum* (May and Herrick 1932; Godin 1953). Abe *et al.* (1962) reported yields of levorotatory malic acid as high as 55 g/100 g of D-glucose for *Aspergillus flavus* and *A. parasiticus*. Iron, manganese, chromium, and aluminum ions also enhance malic acid production.

Despite the ability of fungi to produce malic acid, present commercial production consists of hydrating malic and fumaric acids in the presence of a suitable catalyst and separating the malic acid from the equilibrium product mixture (Irwin *et al.* 1967).

Tartaric, Succinic, Oxalic, and Lactic Acids

Other food acids which may be produced by fungi but are not produced commercially by fungal fermentation processes are tartaric, succinic, oxalic, and lactic. Tartaric acid is used in carbonated beverages. It especially has the ability to augment natural and synthetic grape flavors (Gardner 1968). Tartaric acid is also used in gelatin desserts, fruit jellies, and starch jelly candies (Chichester 1969). Succinic is used in flavoring and as a gelling agent for marmalades (Turi 1969). Oxalic acid is used in the hydrolysis of starch to glucose (Pederson 1971). Lactic acid is used in bakery products, liquid shortening, egg powder, cheese, dried food casein, fruit juice, frozen desserts, manufacture of beer, mincemeat, mayonnaise, and many other food products (Gardner 1968).

Tartaric acid may be produced by *Penicillium notatum*, *Aspergillus niger*, or *A. griseus*, and succinic acid can be produced in yeast fermentations (Pederson 1971). Many species of aspergilli, penicillia, and mucors produce oxalic acid (May and Herrick 1932). Lactic acid has been produced by many species of *Rhizopus*, *Mucor*, and at least one species of *Monilia*. *Rhizopus oryzae* has particularly outstanding ability to produce lactic acid (Prescott and Dunn 1959).

ENZYMES

Fungal enzymes have been used for hundreds of years, especially in the Orient. However, modern industrial enzyme technology probably started with Takamine (1894) and his work with *Aspergillus oryzae*. Today many industrial enzymes are of fungal origin. Those that are in commercial use are listed in Table 13:2.

α -Amylase

α -Amylase, although produced by many species of fungi, is made commercially by either *Aspergillus niger* or *A. oryzae*. Two general methods are used to produce α -amylase by molds. In one method, known as the Takamine process (Takamine 1894), the mold is grown

TABLE 13.2

COMMERCIAL FUNGAL ENZYMES USED IN FOOD PROCESSING

Enzyme	Produced By	Food and Beverage Uses
α -Amylase	<i>Aspergillus oryzae</i> , <i>A. niger</i>	Corn syrup, dextrose, bread and cracker baking, food dextrins, chocolate and licorice syrups
Glucoamylase	<i>Aspergillus awamori</i> , <i>A. niger</i> , <i>Rhizopus niveus</i> , <i>R. delemar</i>	Dextrose, dextrose syrup, baking, degrading gelatinized starch
Pectinases	<i>A. niger</i> , <i>Coniothyrium diplodiella</i>	Clarifying fruit juices and wine, coffee concentration
Waringinase	<i>A. niger</i>	Debittering grapefruit juice
Invertase (sucrase)	<i>Saccharomyces cerevisiae</i> , <i>S. uvarum</i> (formerly <i>S. carlsbergensis</i>)	Confectioneries, liqueurs, cordials, soft-centered chocolates, artificial honey
Lactase (β -D-galactosidase)	<i>A. niger</i> , <i>Kluyveromyces lactis</i> , <i>K.</i> (formerly <i>Saccharomyces</i>) <i>fragilis</i>	Dairy products, upgrading cheese whey
Protease	<i>A. oryzae</i>	Soy sauce, brewing, baking bread, crackers, tenderizing meat
Rennet (rennin, if pure)	<i>Mucor pusillus</i> , <i>M. miehei</i> , <i>Endothia parasitica</i>	Many types of cheese
Glucose oxidase, catalase	<i>A. niger</i>	Powdered egg products, brewing, wines, mayonnaise

on wheat bran in shallow trays. The bran is moistened with water, steam sterilized, and inoculated with spores of *A. oryzae*. After growth, the amylase is extracted from the bran with water. The extract may be concentrated below 50°C to a syrup or the α -amylase may be precipitated from the aqueous extract with alcohol and then dried at 55°C or less. In the other method the fungus, usually *A. niger*, is cultivated by submerged fermentation using a starch-salts medium. The α -amylase is generally recovered by filtering from the mycelium and concentrating the filtrate to a syrup under reduced pressure.

The largest use of fungal α -amylase in food processing is in the production of glucose syrups (Dale and Langlois 1940; Erenthal and Block 1962; Denault and Underkofler 1963; Underkofler et al. 1965). Normally starch is converted with acid until the dextrose equivalent (D.E.) is about 40 to 50. The dextrose equivalent value represents the quantity of reducing sugars formed, calculated as dextrose, and ex-

pressed as a percentage of the total solids. Following the acid conversion, fungal α -amylase is added to increase the D.E. to around 63. With the fungal α -amylase preparations available today, syrups with a high D.E. but low content of dextrose can be formed. These syrups are sweet and have low viscosity and good flavor.

Fungal amylase is also used extensively in the baking industry. Wheat flour does not naturally contain sufficient α -amylase for best baking quality. Fungal amylase from *A. oryzae* is normally added to flour to complement the α -amylase already present in cereals. The combined action of these two amylases supplies maltose for the panary fermentation. Maltose, or some form of fermentable sugar, is required for the yeast to generate carbon dioxide for raising the dough. Fungal α -amylase is preferred over either plant or bacterial α -amylase because it is heat labile and is inactivated early in the baking cycle before the starch gelatinizes (Rubenthaler *et al.* 1965). During baking, heat-stable amylases would act on the gelatinized starch and cause sticky, gummy loaves.

Glucoamylase

Most glucoamylase produced in the United States is from *Aspergillus awamori*. The mold is cultivated in a cornmeal medium by submerged fermentation (Smiley *et al.* 1964). The enzyme is extracellular and is recovered by filtration. The filtrate may be concentrated under a vacuum below 50°C to a thick syrup. For some purposes it is recovered by precipitation with alcohol. The precipitate is dried in the presence of an inert carrier.

In Japan glucoamylase is made from certain *Rhizopus* strains grown on wheat bran. The enzyme is extracted from the fermented bran with water. It has been claimed that the glucoamylase from *Rhizopus* contains less α -amylase and transferase than glucoamylase from *A. awamori*. Because *Rhizopus* glucoamylase is less stable on storage and cannot be produced by submerged fermentation, it has not been used extensively in the United States.

Glucoamylase is widely used in the corn wet milling industry to produce high-dextrose syrups from which crystalline corn sugar is prepared. Some glucoamylase is used in the baking industry. Generally, it accompanies the fungal α -amylase employed by bakers. However, it does have special benefit, since it forms glucose which is readily metabolized by yeast and probably accounts for the improved gassing and loaf volume. Pomeranz *et al.* (1964) and a group at Kansas State University have described the role of glucoamylase in bread manufacture.

Pectic Enzymes

Many fungi produce pectic enzymes, also referred to as pectinolytic

or pectolytic enzymes. The enzyme as produced is actually a complicated mixture of enzymes. The predominant ones are (1) pectin methylesterase, also known as pectin esterase and pectase, which catalyzes the hydrolysis of the methyl ester groups in pectic substances with the formation of methyl alcohol and polygalacturonic acid; and (2) polygalacturonase, also known as pectinase, which degrades pectinic or pectic acid by hydrolysis of the glucosidic linkages of polygalacturonic acid to form smaller polygalacturonates which are water soluble (Endo 1964A,B). Commercial fungal pectinase consists predominantly of polygalacturonase.

Fungi used for commercial pectinase production are generally strains of the *Aspergillus niger* group. In Japan, *Sclerotinia libertiana* is used. Also, the Deuteromycete *Coniothyrium diplodiella* is a good source of pectic enzymes (Endo 1963).

Pectic enzymes are used for clarification of fruit juice, especially apple and grape juice (Aitken 1961; Fuleki and Hope 1964; Endo 1965). Both of these juices contain colloidal polygalacturonates which the enzymes degrade, causing the colloids to coalesce so they can be filtered out. The pectinase not only clarifies the juice but also increases the yield of juice from the crushed fruit.

Fungal pectic enzymes also have been found very useful in clarification of wines (Cruess et al. 1955). In modern practice the enzyme is added to fruit during crushing, which not only clarifies but also increases the yield of juice by as much as 10%. In making red wines, grapes are often crushed warm to extract more color. Unfortunately, this also extracts pectin and makes subsequent clarification more difficult unless fungal pectinases are employed to clarify the must.

Fungal pectic enzymes are also used in processing green coffee beans. The coffee cherry contains a mucilaginous layer surrounding the bean which must be removed before the bean can be dried. Natural fermentation to solubilize the mucilage requires 24 to 48 hr and is often accompanied by undesirable microbial growth which lowers the bean quality. By using fungal pectinases the "fermentation" time is reduced to about an hour and spoilage by microorganisms is forestalled.

Ishii and Yokotsuka (1972) have proposed using pectin transeliminase from *Aspergillus sojae* or *A. japonicus* to clarify fruit juice. Pectin transeliminase solubilizes the polygalacturonate without hydrolyzing the methyl ester groups. Consequently, the juice will contain little or no methanol.

Naringinase

Commercial preparations of naringinase are prepared from cultures of *A. niger*. This enzyme acts on the bitter principle in grapefruit,

naringin, to form nonbitter derivatives (Griffiths and Lime 1959). The enzyme actually consists of a rhamnosidase and a β -glucosidase. Generally the glucosidase is inhibited by glucose naturally present in grapefruit, so that only splitting of rhamnose from naringin occurs. The resulting glucoside, prunin, is not bitter. In addition to naringinase, *A. niger* also produces pectinase which is undesirable in production of grapefruit juice because it destroys the cloudy appearance normally associated with this product. Therefore, the pectinase must be inactivated in some manner, such as by treatment of the enzyme preparation with urea (Thomas et al. 1958).

Invertase

Invertase production is widely distributed among the fungi. Commercial invertase is prepared from *Saccharomyces* yeast strains (Cochrane 1961). The enzyme is found in close association with the yeast cell; in order to free it, the plasmolyzed cells must be treated with a proteolytic enzyme such as papain. Invertase is used to make soft-centered candies by mixing it with the cast cream consisting of sucrose and other ingredients (Janssen 1962, 1963). After coating, the candies are held for 1 to 2 weeks to allow the invertase to reduce the sucrose to invert sugar, which dissolves in the available water and accounts for the liquid center.

Invertase is employed for making artificial honey, for manufacture of liqueurs, frozen desserts, and confections where high sucrose concentrations would tend to crystallize unless converted by invertase to the much more soluble invert sugar.

α -Galactosidase

Like invertase, α -galactosidase is widely distributed in fungi. There are two possible food applications for α -galactosidase. First, it has potential use in sugar refining. Sugar beet juice contains significant quantities of raffinose. Raffinose interferes with sucrose crystal formation from concentrated juice. By treating the juice with α -galactosidase the raffinose is degraded to sucrose and galactose, resulting in increased yield of sucrose crystals. For this purpose the enzyme must be free of invertase for obvious reasons. Fortunately, several fungi do produce significant levels of α -galactosidase free of invertase. *Mortiella vinaciae* (Suzuki et al. 1969) and *Corticium rolfsii* (Kaji and Ichimi 1969) are examples of organisms which produce α -galactosidase with only minor amounts of invertase. The second possible application for α -galactosidase in foods is treatment of soybean "milk" to remove stachyose and raffinose (Sugimoto and VanBuren 1970). These two oligosaccharides have been implicated as causal factors of flatulence associated with consumption of soybeans (Rackis et al. 1970). In this instance the presence of invertase is not

detrimental and fungal enzyme preparations from *A. niger* that are commonly used in food applications can be employed (Smiley et al. 1976).

In both of the above applications the α -galactosidase must be at least partially purified. Because this significantly increases enzyme cost, attempts have been made to conserve enzyme by immobilization on inert supports. Reynolds (1974) immobilized a bacterial α -galactosidase on nylon for the purpose of removing raffinose from sugar beet juice. Smiley et al. (1976) used ultrafiltration membranes in the form of hollow fibers to remove oligosaccharides from soybean milk. The advantage of this method is that the soy proteins are unable to diffuse to the enzyme site and are, therefore, unaffected by any contaminating protease that may be present.

Lactase

Lactose, though available in large quantities in cheese whey, has not found favor as a sugar for food use. It has low solubility which limits quantities that can be added to foods without crystallization. The presence of lactose crystals results in sandy or grainy products. Lactose is not very sweet; this also limits its usefulness. Enzymes have long been known which will effectively hydrolyze lactose to glucose and galactose. These sugars are considerably sweeter and much more soluble than lactose. The high cost of making lactase, coupled with the high cost of treatment, has effectively prevented its use. In the past, the most economical solution to whey disposal was simply to dump it into rivers and streams. With the present emphasis on environmental quality, this manner of disposal will no longer be tolerated. Consequently, new emphasis is being placed on utilizing whey for food use, and lactase can be expected to play a central role in this endeavor.

Lactase is produced by many kinds of microorganisms. Fungal lactase from *A. oryzae* and *A. niger* is well-suited for hydrolysis of whey because the optimum pH range is 4.5 to 5.5, near the normal pH of cheese whey. Pomeranz (1964) has shown that either spray-dried cheese whey or skim milk powder can be used to advantage in bread baking if a fungal lactase is used. The lactose content of these supplements replaces the need for sucrose added to the dough. The glucose resulting from lactose hydrolysis is utilized by the yeast to form carbon dioxide. The galactose, which is not fermented by bakers' yeast, reacts with the wheat proteins during baking to give an excellent crust color. Production of lactase by *Kluyveromyces fragilis* on cheese whey is described in Chap. 6.

Very acceptable dairy products can be made with lactase-hydrolyzed whole and skim milk (Thompson and Brower 1974; Thompson and Gyuricsek 1974). For this purpose a commercial

lactase from *Saccharomyces* (*Kluyveromyces*) *lactis* is used because its optimum pH is near that of milk. Lactase-hydrolyzed milk has been used to make fluid whole milk, milk concentrates, ice cream, spray-dried whole and skim milk, yogurt, cottage cheese, Cheddar cheese and buttermilk. Not only are the lactase-hydrolyzed products acceptable organoleptically, but they can also be consumed by lactose-intolerant individuals (Guy *et al.* 1974). Cheddar cheese made from lactase-hydrolyzed milk ripens faster than cheese from normal milk. This is an important economic consideration for use of lactase-hydrolyzed milk (Thompson and Brower 1974).

Protease

Proteases, like amylases, have a variety of applications. Fungal proteases compete with plant and animal proteases for applications in food processing. In the Orient, proteolytic strains of *A. oryzae* are grown on rice or soybeans to produce koji; this koji is added to vats containing roasted soybeans, cereal grains, and salt brine to make soy sauce. *A. oryzae* protease is also used extensively in bread and cracker formulations where a particularly pliable, extensible dough is required. Addition of protease to doughs also permits sizable reduction in mix times (Coles 1952; Waldt 1965). This is possible because the doughs with protease can be slightly undermixed. The continued action of the enzyme beyond the mixing stage will result in doughs with comparable handling properties to fully mixed doughs without added protease.

Fungal protease is also useful as a chillproofing agent for beer, although normally papain is the enzyme of choice for this application. Fungal proteases have been tested for meat (beef) tenderization and have been found to be particularly effective on muscle fiber but show little action on connective tissue (collagen). Plant proteases (papain, bromelain, and ficin) readily attack connective tissue but show less action on muscle fiber. Therefore, meat tenderizer formulations are mixtures of proteases designed to tenderize particular cuts of beef (Underkofler 1959).

Rennin is traditionally a protease of calf stomach origin and is used universally in cheese manufacture. A growing shortage of calf stomachs has limited the supply of rennin and has spurred search for substitutes. Rennin is peculiar in that after the curd is formed, there is no further action on the casein. Many proteolytic enzymes will form acceptable curd but continuing proteolytic action is detrimental to the cheesemaking process. Some success has been obtained in duplicating calf rennin with enzymes from *Mucor* spp. Commercial rennin substitutes are now offered which are prepared from *Mucor pusillus*.

M. miehei, and *Endothia parasitica*. While enzymes from these fungi offer promise as a rennin substitute, they are still not completely satisfactory from the point of view of flavor and texture (Scott 1973). Acceptable cheese has been made by using mixtures of fungal and calf rennin. Perhaps the future of fungal rennins will be in extending rather than in substituting for calf rennet (mixture of rennins). Sardinias (1976) recently reviewed calf rennet substitutes, giving sources of higher plant, bacterial, and fungal milk-coagulating enzymes.

Glucose Oxidase

Glucose oxidase can be applied to food processing to remove traces of glucose or dissolved oxygen. For instance, if glucose is not removed from commercially dried egg whites, the product will darken on storage due to interaction between the sugar and protein. This nonenzymatic browning results in off-flavors and reduced protein solubility. Also there are losses in whipping properties and foam stability. These latter properties are most important in prepared cake mixes which depend on egg albumin as a functional ingredient.

To remove the glucose, glucose oxidase plus catalase is added to the egg whites (Baldwin et al. 1953). Since an excess of oxygen is required to remove all the glucose, hydrogen peroxide is also added. The catalase breaks down hydrogen peroxide, releasing oxygen. The glucose oxidase then utilizes the oxygen to oxidize glucose to gluconic acid and more hydrogen peroxide. Gluconic acid is not detrimental to the dried egg whites. The procedure can also be used for dried egg yolks as well as whole dried eggs.

Glucose oxidase can be used to remove oxygen from beverages such as white wines, beer, and fruit juices. It is necessary to have excess glucose present to remove all traces of oxygen so that with very dry wines it has been found necessary to add glucose to accomplish removal of oxygen (Ough 1960). It is desirable to remove oxygen from white wine to prevent later color formation and flavor deterioration (Yang 1955).

Most commercial glucose oxidase is isolated from *A. niger* fermentation broths (Swoboda and Massey 1965). One major supplier of glucose oxidase formerly isolated the enzyme from mother liquors of the gluconic acid fermentation. To be useful in food applications, the enzyme must be highly purified. For instance, the presence of protease in glucose oxidase preparations used for desugaring egg white would destroy the albumin. Likewise, in removing oxygen from mayonnaise (Bloom et al. 1956) the presence of amylase in the glucose oxidase would affect the starch that is used to give the product proper body.

Cellulase

Up to now, cellulase has had very limited application in food processing. Its relatively slow action on native cellulose makes it difficult to fit into a food processing operation. Cellulase is composed of at least two enzymes. One, called C_1 , is required for the hydrolysis of insoluble and crystalline cellulose and is the rate-limiting reaction. The other component, known as C_x , is probably, in reality, two β -glucanases. One is an endo enzyme that randomly splits β -1,4 linkages in cellulose chains while the other is an exoenzyme that attacks the cellulose chain from the nonreducing end. Commercial cellulases are of fungal origin. Traditionally, culture filtrates of *A. niger* fermentations were used as their source; *A. niger* cellulase is low in the C_1 component, so its action on insoluble cellulose is very slow. It does work well on amorphous and soluble forms of cellulose such as carboxymethyl cellulose. A more practical source of cellulase is derived from mutant strains of *Trichoderma viride*. This organism gives rise to relatively large quantities of C_1 enzyme and is, therefore, more effective on crystalline cellulose.

Considerable interest has been manifested in recent years in using cellulase to make glucose from the huge amounts of cellulosic waste available annually. The glucose could be used as a source of food and feed as well as a chemical feedstock source for a variety of chemicals now obtained from petroleum. Many basic problems must be solved before cellulase technology will become a major factor as a source of either food or chemicals. A report by Spano et al. (1975) documents the present status of enzymatic hydrolysis of cellulosic waste to glucose.

Lipase

Fungal lipase is not generally produced and added *per se* to foods. Nevertheless, mention should be made of lipase because of its importance in cheese ripening. Italian cheeses such as provolone and Romano depend on lipase from kid rennet paste to provide the proper flavor. Lipase from other sources such as fungal lipase cannot substitute for kid (or sheep) rennet paste. Likewise, rennet extract will not substitute for the paste. Several other cheeses such as Stilton, Gorgonzola, Roquefort, and blue cheese depend on the lipase of *Penicillium roqueforti* for proper flavor development. This is, however, *in situ* action resulting from growth of the mold in the ripening cheese curd.

AMINO ACIDS

Many microorganisms for one reason or another may excrete free amino acids into their growth medium. Advantage is taken of this

property to prepare amino acids for food, feed, and therapeutic purposes. Fungi have not generally been employed commercially for amino acid production, except for lysine. Lysine can be produced in good yields (2.0 g/liter) from a glucose-urea-salts medium by strains of *Ustilago maydis* and *Gliocladium* spp. (Dulaney 1957; Richards and Haskins 1957; Haskins and Spencer 1959). In addition to lysine, the fermentation broth contains glutamic acid and arginine.

Lysine can also be made from α -aminoadipic acid by *Candida utilis* (Sagisaka and Shimura 1957). Broquist et al. (1961) studied lysine production by *Saccharomyces cerevisiae* as well as by *C. utilis*. For highest yields a precursor had to be added to a corn steep liquor medium. Under the proper conditions about 70% of the precursor was converted to L-lysine on a molar basis. In addition, the lysine adheres tightly to the yeast cells so that a lysine-enriched yeast can be readily prepared.

Lysine is used to enrich wheat flour for baking purposes. It can also be added to polished rice to improve the nutritive value of this widely used grain.

In recent years immobilized amino-acylase from *A. oryzae* has been used to prepare L-amino acids from DL-acylamino acids (Tosa et al. 1967). The enzyme is ionically bound to DEAE-Sephadex. Acylamino acids are put through a column containing the enzyme complex where the L-acylamino acid is deacylated to the L-amino acid. The D-form, which is not affected by the enzyme, is racemized chemically and the resulting racemate is recycled. L-Methionine has been made extensively by this procedure in Japan. As an essential amino acid, methionine is used to reinforce vegetable proteins in foods and feeds. However, in the United States synthetic DL-methionine is used, even though twice as much DL as L must be added to give the same nutritional value.

PIGMENTS AND VITAMINS

Fungi are used for fermentative production of β -carotene and riboflavin. β -Carotene is produced by + and - strains of *Choanephora cucurbitarum*, *C. conjuncta*, *Blakeslea trispora*, and *B. circinans*. The medium is rather complicated and contains in addition to cornmeal and cottonseed meal such additives as deodorized kerosene, citrus molasses, and a vegetable oil (Ciegler et al. 1963). Yields of β -carotene of about 1.0 g/liter can be expected. β -Carotene is not being produced by fermentation since, at the present time, synthetic β -carotene is more economical. β -Carotene is widely used as a coloring agent in foods. It is also a source of vitamin A in foods and feeds.

Riboflavin has been produced commercially by the Ascomycetes, *Eremothecium ashbyii* (Rudert 1945) and *Ashbya gossypii* (Malzahn et al. 1959). Yields as high as 5.0 g/liter have been obtained with *A. gossypii* in a medium consisting of corn steep liquor, animal stick liquor, collagen peptone, and vegetable oil. Continuous feeding of glucose was also necessary. Most of the riboflavin produced by these fungi was not purified. The fermentation broth was concentrated, dried, and used as a supplement for animal feeds. The high concentration of riboflavin in the fermentation medium made it easy to isolate and use as a food supplement. Currently most riboflavin is made by chemical synthesis.

The plant hormone gibberellin is made by the mold *Gibberella fujikuroi* (Stodola 1958). The fermentation is quite long and involved and is usually conducted in two stages. The ratios of carbon:nitrogen must be carefully controlled. Yields of around 1.0 g/liter can be obtained in 450 or 500 hr (Borrow et al. 1959). Gibberellin is used as a plant growth regulator for α -amylase in brewery malt and for improving crop yields of cotton, grapes, and celery.

FLAVORINGS OF FUNGAL ORIGIN

Nucleotides

Use.—Flavor potentiators have been used extensively for more than 15 years to enhance the natural flavor of the foodstuff to which they are added. Monosodium glutamate (MSG) in particular has an established place in the potentiator market. However, 5'-inosinate (inosine-5'-monophosphate, IMP) and 5'-guanylate (guanosine-5'-monophosphate, GMP) are useful in improving the flavor of many foods (Nelson and Richardson 1967). Furthermore, Titus (1964) reported that these potentiators are beneficial in soups, gravies, and bouillons at levels of 25 to 100 ppm. They are also useful in improving the flavor of many products containing hydrolyzed proteins and can be used to replace beef extracts in many foods (Kuninaka 1972). Kuninaka et al. (1964) reported a strong synergistic action between MSG and flavor nucleotides. For example:

$$\begin{aligned}10 \text{ g MSG} + 1 \text{ g IMP} &= 55 \text{ g MSG} \\10 \text{ g MSG} + 1 \text{ g GMP} &= 209 \text{ g MSG}\end{aligned}$$

Consequently, combinations of MSG and the nucleotides are used for home cooking, especially in the Orient.

Production.—Monosodium glutamate is produced by neutralization of glutamic acid which can be produced by yeasts and molds.

However, glutamic acid is primarily a fermentation product of the bacterium, *Micrococcus glutamicus*. In contrast, the nucleotides are produced by treating ribonucleic acid (RNA), primarily from yeasts, with nuclease from *Penicillium citrinum* (Kuninaka 1972). The enzyme cleaves the 5'-phosphodiester linkages of the nucleosides to free the flavorful 5'-nucleotides. Also, nuclease from *A. oryzae* and *Streptomyces griseus*, an actinomycete, has been used (Nelson and Richardson 1967).

Yeast Cells

Yeast cells can serve as flavor enhancers or extenders in soups, gravies, sauces, snacks, and cereals. Reportedly, they are excellent carriers for flavorings such as cheese, smoke, barbecue, fish, and chicken. In some formulated meat products use of yeast permits a 20 to 50% reduction in pepper and other spices (Anon. 1974).

Mycelia of Mushrooms and Other Fleshy Fungi

The discovery in the late 1940s that mycelia from fleshy fungi could be efficiently produced in submerged culture led researchers to explore the growth characteristics of several mushroom genera. By manipulating medium composition, temperature, aeration, and other growth factors, investigators have produced mycelia with mushroom-like flavor from *Agaricus bisporus*, *A. blazei*, *Coprinus comatus*, *Tricholoma nudum*, and *Lepiota rachodes*. Mycelia of *Morchella hortensis*, *M. crassipes*, and *M. esculenta* have been found to have a desirable flavor and odor. The United States Food and Drug Administration has approved mycelia of *Morchella* spp. for sale as "morel mushroom flavoring" (Litchfield 1967). At one time, this flavoring was commercially available for use in dehydrated soup sauce and gravy formulations under the trade name Powdered Morel Mushroom Flavorings. Litchfield (1967) stated that this product was competing successfully with dried mushrooms imported from Europe. However, production has since been discontinued according to LeDuy et al. (1974).

Blue Cheese

Nelson (1970) described a submerged fermentation procedure for the production of blue cheese flavor by *Penicillium roqueforti* on a milk-based medium. The resulting flavor contained 7 to 12 times the ketone content of good quality commercial blue cheese and exhibited an efficacy four times that of blue cheese. The product is used commercially in salad dressing, snacks, and party dips where a blue cheese flavor is desired.

FATS AND OILS

Plant seeds are the principal source of fats and oils for edible and technical purposes. Animal fats and fish oil are subsidiary sources. However, as the demand and price increase, it may become feasible to use fungi for fat production. According to Whitworth and Ratledge (1974), *Rhodotorula* and *Lipomyces* spp. of yeast are most favored for fat production; lipid contents range up to 60% of the cell dry weight. Prescott and Dunn (1959) have listed *Penicillium*, *Aspergillus*, *Fusarium*, *Geotrichum*, and *Paecilomyces* spp. of molds as potential fat producers.

The fat from molds in general has a higher content of polyunsaturated fatty acids than that from yeasts and for this reason may have more commercial value (Whitworth and Ratledge 1974). In all cases, about 80% of the lipid content of the cell is triglycerides. The remaining lipids are usually phospholipids, sterols, and some sterol esters (Thorpe and Ratledge 1972). Moreover, different fatty acid compositions can be attained by using different organisms. It also has been found that, when nitrogen is limited, the microbial cell will continue to consume carbon and excessive lipid will accumulate. This flexibility of control over fungal fermentations allows the quality of the lipid to be improved. However, the successful production of fats by fungi will depend upon the abundance of a cheap and easily available substrate.

MANNITOL

Mannitol is generally prepared as a by-product of sorbitol manufacture. It can, however, be made by fermentation of glucose with *Aspergillus candidus* (Smiley et al. 1967). The organism requires sodium nitrate, an organic nitrogen source such as yeast extract, and daily feeding of glucose to obtain maximum mannitol yields. If a constant glucose source is not supplied, the organism utilizes the mannitol present and yields will be lowered.

Mannitol is used as a humectant and bulking agent in foods. Specifically, it is used in soybean chewing gums, sugarless hard candy, and sugarless chocolate.

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